Expression of Epstein-Barr Virus Latent Membrane Protein-1 in Hodgkin and Reed-Sternberg Cells of Classical Hodgkin’s Lymphoma: Associations with Presenting Features, Serum Interleukin 10 Levels, and Clinical Outcome1

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ABSTRACT
Purpose: EBV-latent membrane protein-1 (LMP-1) is often expressed in Hodgkin and Reed-Sternberg (HRS) cells of classical Hodgkin’s lymphoma (cHL), but its clinical significance is controversial. We correlated LMP-1 with presenting features, including serum interleukin 10 levels and clinical outcome.

Experimental Design: Patients were eligible if they had biopsy-proven cHL, were untreated, HIV-1 negative, and had available archival tissue. LMP-1 expression was determined by immunohistochemistry.

Results: We identified 577 patients with cHL with a median age of 30 years, 55% of whom were male. LMP-1 was expressed in HRS cells of 124 patients (21%) and was detected in 78 of 461 (17%) patients with nodular sclerosis compared with 44 of 112 (39%) with mixed cellularity (P < 0.001 by Fisher’s exact test). Patients with tumors with LMP-1-positive HRS cells had higher serum interleukin 10 levels (P = 0.009 by Mann-Whitney test). For the 303 patients treated with doxorubicin, bleomycin, vinblastine, and dacarbazine or equivalent regimens, the 5-year failure-free survival (FFS) for those with LMP-1-positive tumors was 74% compared with 81% for those with LMP-1-negative tumors (P = 0.23, by log-rank test). Overall survival (OS) at 5 years for patients with LMP-1-positive tumors was 90 versus 91% for patients with LMP-1-negative tumors (P = 0.8 by log-rank test). Expression of LMP-1 was not associated with different FFS and OS in patients treated with other regimens or with radiotherapy alone.

Conclusions: LMP-1 was expressed by HRS cells in 21% of CHL and correlated with mixed cellularity type and higher serum interleukin 10 levels. The presence of LMP-1 was not associated with FFS or OS in uniformly treated patients.

INTRODUCTION
Recent molecular studies have demonstrated that HRS3 cells are derived from germinal center B cells that have either transcriptional defects or dysfunctional rearrangements of the immunoglobulin genes and, therefore, lack surface B cell receptors (1–4). In normal B cells, these defects would be expected to cause apoptosis. Therefore, in HL, HRS cells appear to have developed mechanisms to prevent apoptosis.

Several investigators have shown that HRS cells are often latently infected by EBV and express several EBV-encoded viral proteins (5–7). One of these is LMP-1, which can immortalize B cells in vitro, potentially by induction of the antiapoptotic bcl-2 gene (8–10). It is therefore possible that LMP-1 expression by HRS cells may block apoptosis and contribute directly or indirectly to the development of HL.

Cellular IL-10 also has been detected in HRS cells and reactive lymphocytes by in situ hybridization or immunohistochemical methods (5, 6, 11, 12). The immunosuppressive prop-

1 The abbreviations used are: HRS, Hodgkin and Reed-Sternberg; HL, Hodgkin’s lymphoma; cHL, classical HL; LMP-1, latent membrane protein-1; IL, interleukin; ABVD, Adriamycin (doxorubicin)-bleomycin-vinblastine-dacarbazine; MOPP, nitrogen mustard-vincristine-procarbazine-prednisone; CHLVPP/PABIOE, chlorambucil-vinblastine-procarbazine-prednisolone/prednisolone; Adriamycin (doxorubicin)-bleomycin-Oncovin (vincristine)-etoposide; EBER, Epstein-Barr virus-encoded RNA.

2 To whom requests for reprints should be addressed, at Department of Hematopathology, Box 72, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 794-5446; Fax: (713) 745-0736; E-mail: jnmediero@mdanderson.org.
erties of IL-10 may shield LMP-1-positive HRS cells from immunological rejection (13, 14). In addition, IL-10 can also up-regulate bcl-2 expression and protect B cells from apoptosis (15). This may account for the association of elevated serum IL-10 levels with inferior clinical outcome in patients with cHL (16–19).

These data suggest that LMP-1 expression or associated expression of cellular IL-10 by HRS cells may contribute to their survival by protecting HRS cells from spontaneous and chemotherapy-induced apoptosis (20–22). On the basis of these considerations, we investigated the expression of LMP-1 in HRS cells of previously untreated patients with cHL and correlated the findings with presenting clinical and laboratory features, including serum IL-10, as well as clinical outcome. To eliminate the confounding effect of variable treatment, we used FFS as an end point in patients treated with equivalent regimens.

PATIENTS AND METHODS

Patients. Patients were eligible if they had a histologically confirmed diagnosis of cHL and no prior treatment when they presented from 1984 to 2000. The patients were treated at The University of Texas M. D. Anderson Cancer Center (Houston, TX); Istituto Nazionale Tumori, (Milan, Italy); University of Verona (Verona, Italy); and the National and Kapodistrian University of Athens (Athens, Greece). Patients were selected on the basis of tissue blocks being available for immunohistochemical determination of LMP-1. The histological diagnosis was confirmed by review of available slides at the time LMP-1 expression was evaluated, according to criteria defined by the WHO classification (23). In all cases, the neoplastic cells were positive for CD30 and/or CD15. Patients with positive antibody titers to HIV-1 were excluded from analysis.

Staging and Therapy. Details of the clinical staging, laboratory features and therapeutic regimens of this patient population have been described previously (24, 25). For analysis of clinical outcome, ABVD, epirubicin-bleomycin-vinblastine-dacarbazine, cyclophosphamide-vinblastine-prednisone-procarbazine/Adriamycin (doxorubicin)-bleomycin-dacarbazine-prednisone-carmustine, MOPP/ABVD, and etoposide-epirubicin-bleomycin-cyclophosphamide-prednisone were considered equivalent regimens (16, 26).

Complete remission was defined as absence of disease for at least 1 month as determined by physical examination and appropriate laboratory and imaging studies. Partial remission was defined as >50% reduction of tumor mass measurable in two dimensions and lasting at least 1 month. Progressive disease was defined as enlargement (>25%) of a preexisting site of disease or development of disease in a previously uninvolved site. Primary treatment failure was defined as PD during initial treatment as well as failure to achieve CR or PR after initial therapy. Relapse was defined as progression occurring after achievement of CR or PR.

Immunohistochemical Methods. To assess for LMP-1 expression, slides were incubated for 1 h with a monoclonal antibody cocktail specific for LMP-1 (clone CS-1-4; Dako, Carpinteria, CA) in a dilution of 1:50 in 0.1% BSA in 50 mM Tris-HCl buffer (pH 7.6). The remaining immunohistochemical protocol has been described previously (24). The EBV-positive cell line B95-8 (27) and K562 cells were used as external positive and negative controls, respectively.

Evaluation of all immunostained slides was performed without knowledge of the clinical outcome. Slides were considered evaluable for LMP-1 if external controls stained appropriately. Any cytoplasmic LMP-1 staining of HRS cells was considered positive.

Serum IL-10 Levels. Sera obtained at the time of the initial clinic visit, that had never been thawed, were used to determine human IL-10 levels with an antigen capture ELISA kit (Quantikine; R&D Systems, Minneapolis, MN) as described previously (16). The upper normal limit for serum IL-10 levels, derived from healthy volunteers, was 10 pg/ml (16).

Statistical Analysis. FFS was measured from the beginning of treatment to primary treatment failure, relapse, or last follow-up. Patients who died during treatment without evidence of progressive disease, or after the end of therapy without prior evidence of relapse were censored. OS was measured from the time of diagnosis to the time of death from any cause or to the time of last follow-up. The actuarial probability of FFS and OS was estimated according to the method of Kaplan and Meier (28). The statistical significance of differences in FFS between patient groups was estimated by the log-rank test (29). Multivariate survival analysis was based on Cox’s proportional haz-

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Table 1

<table>
<thead>
<tr>
<th>Histology</th>
<th>Patients tested</th>
<th>LMP-1-positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>No. %</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td>462</td>
<td>78 17</td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td>112</td>
<td>44 39 &lt;0.001</td>
</tr>
<tr>
<td>Lymphocyte depletion</td>
<td>3</td>
<td>2 67</td>
</tr>
<tr>
<td>All patients</td>
<td>577</td>
<td>124 21</td>
</tr>
</tbody>
</table>

*The difference in the percentage of LMP-1-positive tumors between mixed cellularity and the other histologies (nodular sclerosis + lymphocyte depletion) was evaluated by Fisher’s exact test.
The association between LMP-1 expression and presenting clinical or laboratory features was evaluated by the \( t^2 \) and Fisher's exact tests. Nonparametric Mann-Whitney test was used to correlate patient age or serum IL-10 levels with LMP-1 expression. All statistical calculations were performed using StatView (Abacus Concepts, Inc., Berkeley, CA).

**RESULTS**

**Patient Population.** We identified 1686 untreated patients with cHL who presented to the participating institutions between 1984 and 2000. Archival biopsy material was available for 577 patients who constituted the study group. The clinical and laboratory features of these patients at the time of diagnosis have been reported earlier (24, 25). Treatment was ABVD or equivalent regimens in 303 patients, Novantrone (mitoantrone)-Onocovin (vincristine)-vinblastine-prednisone and radiotherapy in 144, MOPP in 51, radiotherapy alone in 60, and various other regimens in 19 patients (24, 25, 31, 32).

**LMP-1 Expression.** LMP-1 was detected in HRS cells of cHL in 124 of 577 (21%) patients (Table 1). When detected, most HRS cells of a given tumor were LMP-1 positive; the median percentage of positive HRS cells was 94% and ranged from 60 to 100% (mean \( \pm \) SD, 91.3 \( \pm \) 8.8%; Fig. 1). Staining intensity was comparable among HRS cells in most LMP-1-positive tumors in well-stained areas. LMP-1 expression was significantly more frequent in mixed cellularity, 39%, than nodular sclerosis, 17% (\( P = 0.001 \) by Fisher's exact test). As indicated in Table 2, LMP-1 was expressed in cHL in 30% of patients younger than 16 years and in 32% of patients older than 45 years compared with 19% in the patients 16–45 years (\( P = 0.008 \); \( \chi^2 \) test). Patients with LMP-1-positive cHL more often presented with Ann Arbor stage other than II (\( P = 0.001 \); \( \chi^2 \) test), had inguinal or iliac node involvement (\( P < 0.0001 \); Fisher's exact test), or elevated serum \( \beta_2 \)-microglobulin levels (\( P = 0.01 \); Fisher's exact test). By contrast, LMP-1 expression was detected in 10% of patients with large mediastinal masses compared with 24% for all other patients (\( P = 0.005 \); Fisher's exact test).

**Association of LMP-1 Expression with Serum IL-10 Levels.** Sera were available for IL-10 analysis in 230 patients, 142 of whom were treated with ABVD or equivalent regimens. When serum levels of IL-10 were analyzed as a continuous variable, they were significantly higher in patients with tumors expressing LMP-1 in HRS cells (\( P = 0.009 \) by Mann-Whitney test).
When IL-10 was analyzed as a categorical variable using 10 pg/ml as a cutoff, elevated serum levels of IL-10 were detected in 19 of 41 (46%) patients with LMP-1-positive tumors compared with 48 of 189 (25%) patients with LMP-1-negative tumors. Multivariate analysis was restricted to the 156 patients with stage I or II disease, the 5-year FFS for patients with LMP-1-positive tumors was 83%, respectively (87 versus 85%; \( P = 0.9 \)). Similarly, among the 147 patients with stage III or IV disease, OS at 5 years was not significantly different (87 versus 85%; \( P = 0.9 \)) for patients with LMP-1-positive versus -negative tumors. Multivariate analysis using Cox’s proportional hazards model did not reveal any independent prognostic contribution attributable to LMP-1 expression for either FFS or OS in patients treated with ABVD or equivalent regimens (data not shown).

Thirty-one of 144 (22%) patients with Ann Arbor stage I–III disease who were treated with Novantrone (mitoantrone)-Onocovin (vincristine)-vinblastine-prednisone and radiotherapy had refractory disease or relapsed. The 5-year FFS for patients with LMP-1-positive versus LMP-1-negative tumors was 75 and 83%, respectively (\( P = 0.54 \)). Eleven of 60 (18%) patients treated with primary curative radiotherapy failed. The 5-year FFS was 81% for patients with LMP-1-positive tumors versus 80% for patients with LMP-1-negative tumors (\( P = 0.8 \)).

**DISCUSSION**

We report that the EBV-encoded oncogenic protein LMP-1 was expressed by HRS cells in 21% of cHL in a predominantly adult patient population. LMP-1 expression was more frequent in mixed cellularity type and in tumors of patients who were younger than 16 or older than 45 years. LMP-1 expression was also more common in patients with inguinal or iliac lymph node involvement and in patients with elevated serum \( \beta_2 \)-microglobulin levels (Table 2). LMP-1 expression was also associated with significantly higher serum levels of IL-10. Expression of LMP-1 was less frequent in HRS cells of patients with large mediastinal masses and in patients with Ann Arbor stage II (Table 2). The higher frequency of LMP-1 expression in tumors of younger patients has been reported previously (33, 34). The association with Ann Arbor stage is most likely explained by the less frequent expression of LMP-1 in nodular sclerosis, which is more common in younger patients who have supradiaphragmatic tumors (\( P = 0.8 \); Fig. 4). Among the 156 patients with stage I or II disease, the 5-year OS was 95% for patients with LMP-1-positive tumors and 96% for patients with LMP-1-negative tumors (\( P = 0.7 \)). Similarly, among the 147 patients with stage III or IV disease, OS at 5 years was not significantly different (87 versus 85%; \( P = 0.9 \)) for patients with LMP-1-positive versus -negative tumors. Multivariate analysis using Cox’s proportional hazards model did not reveal any independent prognostic contribution attributable to LMP-1 expression for either FFS or OS in patients treated with ABVD or equivalent regimens (data not shown).

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motic disease, Ann Arbor stage II with bulky mediastinal masses, and lower serum β2-microglobulin levels (35, 36). By contrast, the more common association with inguinal or iliac lymph node involvement may reflect the relatively more frequent association of mixed cellularity type with infradiaphragmatic cHL. In this study, LMP-1 expression by HRS cells was not associated with inferior FFS or OS in patients treated with equivalent regimens. The lack of prognostic significance of LMP-1 seen in the univariate analysis was confirmed by multivariate analysis with Cox’s proportional hazards model.

There are conflicting reports in the literature regarding the clinical significance of latent EBV infection in HRS cells of cHL (Refs. 37–43; Table 3). These studies vary in methods of patient accrual, EBV detection, treatment regimens, and end point of analysis. Most studies included adults of both sexes, but one included only females (42). Treatment was uniform in only two studies, each of which analyzed few patients (38, 41). The detection method of latent EBV infection also varied. In two reports of patients with uniform treatment, LMP-1 was detected immunohistochemically in one (41), and EBV small encoded RNA was detected by in situ hybridization in the other (38). End points also differed with OS being most common (Table 3). However, OS is affected not only by presenting patient characteristics and initial therapy but also by the postrelapse salvage therapy and by the natural limitation of life expectancy. Because initial therapy was variable in most of these reports and salvage therapy was not even mentioned, OS is a problematic end point. Disease-free survival was used in two studies (39, 41) but does not account for primary treatment failures. Finally, FFS as defined by Murray et al. (38), included toxicity and deaths from other causes as events.

In one series of 100 patients with uniform treatment (MOPP/ABVD), LMP-1 expression was not associated with a statistically different OS or disease-free survival (41). Our results are in agreement with this study, with the advantages of a large study group of 303 patients and analysis of FFS with censoring of events other than cHL relapse or failure. By contrast, the studies reporting significant differences in prognosis for patients with EBV-positive or -negative HL (37, 39, 40, 42) are confounded by variable therapy, variable use of EBV detection methods, and the use of OS as the end point. These methodological factors may account, in part, for the conflicting reports on the prognostic significance of latent EBV infection in HRS cells (Table 3). However, it is also possible that the prevalence of different EBV strains in different populations (44) or mutations in the LMP-1 gene (45), each associated with more aggressive disease (46), may also contribute to the variability in prognosis among these different patient populations.

In this study, LMP-1 expression was associated with elevated serum levels of IL-10 (Fig. 2). As elevated serum IL-10 levels predict poor prognosis (16–19), it is surprising that LMP-1 positivity did not also correlate with adverse outcome. However, because of the low frequency (21%) of LMP-1 expression in the whole patient population, 19 (46%) of the patients with LMP-1-positive tumors had elevated serum IL-10 levels compared with 48 (25%) patients with LMP-1-negative cHL. The source of serum IL-10 in the latter patients is uncertain but is probably attributable to bystander-reactive lymphocytes (12). Others have reported that HRS cells that lack LMP-1 rarely express IL-10 (5, 6, 11, 12). Because we did not study the cases in this study for IL-10 using immunohistochemistry, we cannot assess the relationship between LMP-1 and IL-10 in HRS cells.

The expression of LMP-1 in HRS cells may have therapeutic implications. Immunotherapy has already targeted viral proteins expressed by latently infected HRS cells, but the results are not definitive (47). Lymphoblastoid B cells expressing LMP-1 are susceptible to LMP-1 antisense oligonucleotides in vitro (48, 49). However, these agents have yet to be used in clinical trials.

In conclusion, our study showed that LMP-1 is detected in HRS cells of 21% of patients with cHL. LMP-1 expression is not associated with statistically different FFS and OS in uniformly treated patients with cHL.

ACKNOWLEDGMENTS

We thank Mary K. Laqua for excellent technical assistance.

REFERENCES


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